

# Conditions for Competence in the *Bacillus licheniformis* Transformation System

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Some variables in the *Bacillus licheniformis* transformation system, such as inoculum size, initial cell density in transformation medium, and time of optimal competence, have been explored, and a methodology has been developed to obtain greater reproducibility and maximal competence for transformation. The transformation system in *B. licheniformis* has been shown to be similar to that of *B. subtilis* in a number of respects, including "dormancy" of transformed cells and the concentration of transforming deoxyribonucleic acid required for saturation.

Although transformation of *Bacillus licheniformis* was initially reported in 1964 by Gwinn and Thorne (5) and Leonard et al. (8), the system has not been extensively used as a genetic tool primarily because extended times of incubation with transforming deoxyribonucleic acid (DNA) and high concentrations of DNA were required to obtain appreciable transformation. Methods developed in the present study utilize a "step-down" during transfer from growth to transformation medium. This procedure has been found useful for the development of competence in other transformation systems. Salts composition of the media, techniques initially employed, and bacterial strains are those of Leonard et al. (8, 9). The methodology finally developed gives reproducible and improved transformation, with times of exposure to DNA and concentration of DNA reduced to a level comparable with those required by the closely related organism *B. subtilis* 168.

## MATERIALS AND METHODS

**Bacterial strains.** The recipient culture in all transformation experiments originated from a single-colony isolate of *B. licheniformis* strain 9945A-M17R-1 from the collection of C. Leonard (designated by Leonard as Cap<sup>-</sup>Ad<sup>-</sup>).

**Chemicals.** Medium was routinely prepared from analytical reagent grade chemicals (Fisher Scientific Co., Pittsburgh, Pa., or Mallinkrodt Chemical Works, St. Louis, Mo.) with the use of deionized water. Acid casein hydrolysate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and agar, peptone, Tryptose Blood Agar Base, and Penassay medium were products of Difco.

A-50m spherical agarose for columns, Pronase B grade, adenosine, and guanosine were obtained from Calbiochem, Los Angeles, Calif.; inosine, from Mann

Research Laboratories, New York, N.Y.; and ribonuclease, from Worthington Biochemical Corp., Freehold, N.J.

Lysozyme grade I from Sigma Chemical Co., St. Louis, Mo., sodium lauryl sulfate from Fisher Scientific Co., and sodium deoxycholate from Nutritional Biochemicals Corp. were used in preparation of DNA.

**Media.** Growth medium contained the following, per liter: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 g; KH<sub>2</sub>PO<sub>4</sub>, 5.5 g; K<sub>2</sub>HPO<sub>4</sub>, 14.7 g; sodium citrate·2H<sub>2</sub>O, 3.0 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.0 g; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.02 g; CaCl<sub>2</sub>, 0.113 g; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.16 g; L-tryptophan, 0.1 g; peptone, 0.5 g; acid casein hydrolysate, 5.0 g; glucose, 5.0 g; and adenosine, 0.01 g.

Transformation medium contained the following, per liter: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 g; KH<sub>2</sub>PO<sub>4</sub>, 5.5 g; K<sub>2</sub>HPO<sub>4</sub>, 14.7 g; sodium citrate·2H<sub>2</sub>O, 3.0 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 3.23 g; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.151 g; peptone, 2.0 g; and adenosine, 0.01 g.

Transformation medium plus NaCl contained nine volumes of transformation medium (above) plus one volume of 4 M NaCl, to give a final concentration of components nine-tenths of that indicated above for transformation medium plus 0.4 M NaCl. (Later addition of one-tenth volume of transforming DNA in 2 M NaCl brought the final concentration of NaCl to 0.6 M.)

Dilution salts contained, per liter: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 g; KH<sub>2</sub>PO<sub>4</sub>, 5.5 g; K<sub>2</sub>HPO<sub>4</sub>, 14.7 g; sodium citrate·2H<sub>2</sub>O, 3.0 g; and MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.20 g.

Agar overlay tubes contained, per liter: agar, 5.0 g; acid casein hydrolysate, 0.5 g; and glucose, 5.0 g. Agar plates contained, per liter: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0 g; K<sub>2</sub>HPO<sub>4</sub>, 14.0 g; KH<sub>2</sub>PO<sub>4</sub>, 6.0 g; Na citrate·2H<sub>2</sub>O, 1.0 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.20 g; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.198 g; glucose, 5.0 g; and casein hydrolysate, 0.5 g. When plating for viable cells rather than Ad<sup>+</sup> transformants was carried out, approximately 200 μg of adenosine was added to the plate before spreading or to the overlay agar tube before pouring. In some experi-

ments, viable-cell counts were obtained by plating on Tryptose Blood Agar Base plates.

**Spore suspensions.** Spore suspensions used for routine inoculation of growth medium cultures were prepared as follows. Cells from an isolated single colony were used to inoculate a liquid culture of Schaeffer's broth (11). The culture was shaken at 37 C for 2 to 4 days until the bulk of the culture was present as spores which were removed by centrifugation. The pellet was washed twice before resuspension in one-tenth volume of dilution salts. Samples in screw-capped tubes were stored frozen at -20 C. A single sample was thawed as necessary and diluted approximately 50 times with water; clumped spores were removed by decantation. The decanted supernatant liquid containing suspended spores was stored at 4 C during a period of routine use, and care was taken not to allow extended exposure to room temperature, at which some germination occurred even in water suspensions. The spore suspension was mixed vigorously just prior to each use for inoculation of growth medium.

**Transforming DNA.** DNA prepared from the wild-type strain 9945A Ad<sup>+</sup> Cap<sup>+</sup> was used to obtain an Ad<sup>+</sup> Cap<sup>-</sup> transformant of the recipient Ad<sup>-</sup> Cap<sup>-</sup> culture. DNA prepared (as below) from this Ad<sup>+</sup> Cap<sup>-</sup> transformant was then routinely used as donor DNA in subsequent experiments. This procedure avoided capsule formation which otherwise occurred in a large proportion of Ad<sup>+</sup> transformants and increased the difficulty in obtaining accurate plate counts.

**Preparation of donor DNA for transformation experiments.** Cultures in Difco Penassay medium were grown overnight at 37 C. The next morning, a 20-fold dilution was made into fresh medium and the turbidity of the culture (Klett-Summerson color-meter, filter no. 56) was followed for approximately 3 hr during growth at 37 C. Cells in exponential growth phase were harvested by centrifugation and resuspended in saline-citrate plus 20% sucrose. Lysozyme (500 µg/ml) was added, and the cell suspension was incubated at 37 C for 1 to 2 hr, until the bulk of the culture was converted to protoplasts as revealed in a phase-contrast microscope. Protoplasts were removed by centrifugation and lysed by resuspension in saline-citrate minus sucrose. The suspension was incubated for 30 min with 50 µg of ribonuclease per ml, followed by the addition of 0.3% sodium lauryl sulfate, which caused clearing of the suspension. After incubation with 0.7 to 1.0 mg of Pronase per ml (heated to inactivate nucleases) for 1 to 2 hr at 45 C, the sample was chilled in ice and the DNA was precipitated by the addition of two volumes of cold 95% ethyl alcohol. Precipitated material was transferred to 2 M NaCl + 0.2 M citrate and treated in succession with ribonuclease (60 min at 37 C) and Pronase (1 to 3 hr at 45 C). Finally, 0.1% sodium deoxycholate was added and, after incubation for 1 to 2 hr at 45 C, the sample was refrigerated overnight. The following day, turbid material was removed by centrifugation and the DNA obtained by ethyl alcohol precipitation was dissolved in 2 M NaCl. After a second deoxycholate treatment, turbid material was removed by centrifugation and

DNA obtained by a final ethyl alcohol precipitation was dissolved in 2 M NaCl and stored at 4 C for future use.

**Growth and transformation procedures.** A small volume of a suspension of spores of the recipient Ad<sup>-</sup> Cap<sup>-</sup> culture was inoculated into 10 ml of growth medium in a 125-ml baffle-bottom Erlenmeyer flask (Bellco Glass Inc., Vineland, N.J.). The culture was shaken at 37 C overnight on a metabolite shaker (model G77; New Brunswick Scientific Co., New Brunswick, N.J.) at 125 rev/min. The overnight culture containing large particles of clumped cells was harvested by centrifugation, washed once with 5 ml of transformation medium, and resuspended by Vortex mixing in 3.0 ml of transformation medium. Particles of clumped cells not resuspended by this procedure were allowed to settle to the bottom of the tube for 1 to 2 min, and the top fraction containing resuspended cells was diluted into transformation medium plus NaCl. [The clumping tendency of the Ad<sup>-</sup> Cap<sup>-</sup> culture in both growth and transformation media was reduced by the addition of other nucleic acid bases (guanosine, inosine), apparently without reduction of subsequent transformation.] Competence developed equally well whether small portions of culture were shaken in tubes or larger volumes were shaken in flasks. At the expected time of competence, one-tenth volume of DNA in 2 M NaCl was added and shaking was continued. After the desired time of exposure to DNA, dilutions and platings were carried out to determine viable cells and Ad<sup>+</sup> transformants present in the culture. Originally, dilutions were made by pipetting into tubes containing dilution salts and suitable samples were then spread on plates. However, the yield of transformants was increased when handling procedures were altered and dilutions were made by use of calibrated loops and dilution tubes containing melted agar overlay (maintained at 45 C). Contents of the final agar overlay dilution tubes were poured onto plates which were rocked gently to allow even layering. Plate counts of duplicate samples were reproducible, although some degree of cell clumping in transformation medium plus NaCl was always apparent microscopically. Absolute values for per cent transformation therefore should be accepted with some caution.

**Assay methods.** The T<sub>m</sub> of DNA preparations was determined by the method of Marmur and Doty (10). DNA was measured colorimetrically by the diphenylamine method of Burton (2), and RNA was determined by the orcinol method of Dische (3).

## RESULTS

Washed spores rather than vegetative cells were used as an inoculum for growth medium, since these were assumed to be less subject to variations resulting from previous growth conditions and harvesting techniques. Spore suspensions, therefore, were routinely used in spite of the fact that preliminary experiments indicated that a higher level of transformation might be obtained through the use of vegetative-cell inocula. Provided that

extended periods of exposure to room temperature were avoided, spore suspensions gave reproducible results over several months of use.

Preliminary experiments were first carried out to determine an optimal inoculum size and its relation to the growth period. Three sets of growth medium flasks were inoculated with various volumes of spore suspension and harvested after a growth period of 17, 20, or 23 hr. The cells from each culture flask were treated identically, as described in Materials and Methods, and competence for transformation was measured 380 min after resuspension in the transformation medium. Figure 1 presents the results which indicated that for each growth period a different size of spore inoculum was required to obtain maximal competence, the shortest growth period requiring the largest inoculum and vice versa.

A study was next carried out to determine the relationship between initial cell density in transformation medium and the time at which maximal

competence occurred. For this purpose, cells were grown for a period of 17 hr after inoculation with the volume of spore suspension previously found optimal. After growth, the culture was harvested by centrifugation, washed, and resuspended in transformation medium without NaCl. Cultures were started in transformation medium plus NaCl at initial densities of approximately  $7.5 \times 10^6$ ,  $3.5 \times 10^6$ , and  $1 \times 10^5$  cells/ml. Competence for transformation was measured in each of the cultures at intervals after dilution. Maximal competence was reached at widely separated time intervals, as can be seen in Fig. 2. The culture with the greatest initial cell density reached maximal competence earliest (at about 200 min), whereas cells of intermediate density became competent only at a considerably later time (after 400 min). Cells resuspended at the lowest initial density did not show appreciable competence in the time period used in this experiment (465 min).

Earlier reports of transformation in *B. licheni*-

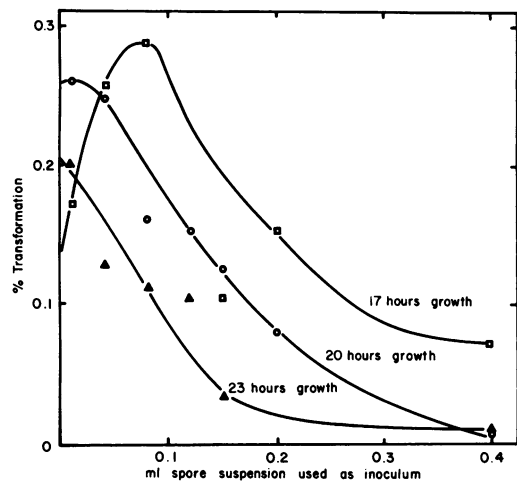


FIG. 1. Relationship of spore inoculum size, growth period, and transformability. Varying samples of *Ad*-spore suspension were used to inoculate a series of flasks in triplicate. A set of flasks was placed on a shaker at 37°C at three different times, and all flasks were harvested at the same time after overnight growth. Total growth period of the first set of cultures was 23 hr; for the second set, 20 hr; and for the third set, 17 hr. After the initial growth period, all cultures were treated identically. A 10-ml amount of culture was centrifuged. The cell pellet was washed with 5 ml of transformation medium and then resuspended in 3 ml of transformation medium. A 50 times dilution of suspended cells was made into transformation medium plus NaCl, and transformation was tested at 380 min by 30-min exposure to DNA. *Ad*<sup>+</sup> transformants and total viable cells were determined by plating, and the per cent transformation for each flask was plotted against the volume of spores used as inoculum.

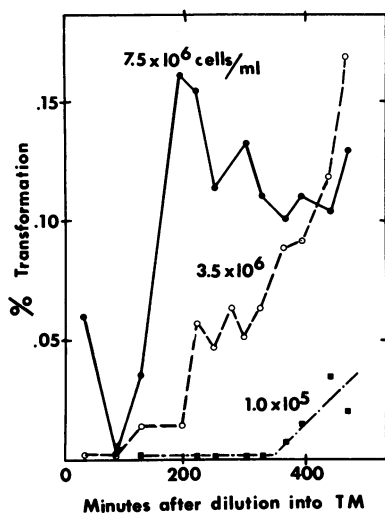


FIG. 2. Relationship of initial cell density to time of maximal competence. A 10-ml culture of the *Ad*-recipient was grown for 17 hr, and, after washing (5 ml) and resuspension (3 ml), 20-, 50-, and 1,000-fold dilutions of the suspended cells were made into transformation medium plus NaCl. Samples (0.45 ml) of the three dilutions were pipetted into a series of tubes which were shaken at 37°C, and at various time intervals DNA was added to a tube of each dilution. At 15 min after addition of DNA, dilutions were made, and viable cells and *Ad*<sup>+</sup> transformants were determined by plating. The per cent transformation obtained is plotted against resuspension time in transformation medium plus NaCl. Viable-cell counts obtained immediately after resuspension in transformation medium plus NaCl indicate that the initial cell density of the 20-, 50-, and 1,000-fold diluted cultures was  $7.5 \times 10^6$ ,  $3.5 \times 10^6$ , and  $1 \times 10^5$  cells/ml, respectively.

*formis* indicated that no cell division occurred during the first 3 to 4 hr of suspension in transformation medium (8, 12). However, this was not the case with the modified media used here. Growth curves obtained by viable counts showed a generation time which varied between 45 and 75 min. Division continued until a cell density of about  $10^8$  to  $2 \times 10^8$  cells/ml was reached. The data of the experiment in Fig. 2 (above) showed no readily discernible correlation of time of competence with growth rate, maximal cell density reached, or cell density at the time of maximal competence.

The degree of competence achieved by a culture was found to be considerably influenced by temperature shifts (which were found harmful) and by small changes in the washing and resuspension procedures used during transfer of cells from growth to transformation medium. For example, if the transformation medium used to wash cell pellets contained an additional 0.4 M NaCl, transformation was reduced as much as 50% compared with a control washed with transformation medium minus additional NaCl. This appears paradoxical in view of the fact that the next procedure after resuspension in each case involved dilution into transformation medium plus 0.4 M NaCl. The effect may be related to cell density at the time of exposure to NaCl. On the other hand, for a given density of cells in transformation medium, the time at which NaCl was added did not appear to be important. The optimal concentration of NaCl was found to be

0.6 to 0.8 M, regardless of whether the additional salt was present at the time of initial resuspension in transformation medium or was added just prior to the addition of DNA at the period of maximal competence.

Minor variations in growth supplements or dilution and plating techniques were also found to have a serious effect on the per cent transformation obtained. This was demonstrated in an experiment designed to test the relative sensitivity of transformants and total viable cells to osmotic shock. Two cultures were used in this experiment. In culture I, guanosine was substituted for the adenine as growth requirement of the organism, whereas guanosine monophosphate was used in culture II. After the cultures reached competence and were exposed to transforming DNA, two techniques were used in plating. In the first (control) method, all dilutions prior to plating were made into tubes of overlay agar. In the second (osmotic shock) method, the initial dilution tube contained water instead of agar. As the data in Table 1 show, in culture I only 6% of the viable cells were lost through osmotic shock, although 52% of the Ad<sup>+</sup> transformants were lost. Culture II was apparently more sensitive to osmotic shock; a surprisingly large proportion of the viable-cell count was lost (60%), and Ad<sup>+</sup> transformants were decreased by 73%. Since in each case the newly transformed cells were more sensitive than the bulk of the population, the calculated per cent transformation was appreci-

TABLE 1. Osmotic shock effects on viability<sup>a</sup>

Culture <sup>b</sup>	Growth supplement	Osmotic shock step	Total viable cells/ml	Ad <sup>+</sup> transformants/ml	Per cent transformation
I	Guanosine	—	$9.62 \times 10^7$	$6.78 \times 10^6$	0.705
		+	$9.01 \times 10^7$ (-6.04%) <sup>c</sup>	$3.29 \times 10^6$ (-51.5%)	0.367 (-48%)
II	Guanosine monophosphate	—	$1.65 \times 10^8$	$1.68 \times 10^6$	1.02
		+	$6.60 \times 10^7$ (-60%)	$4.56 \times 10^6$ (-72.8%)	0.691 (-32.4%)

<sup>a</sup> Cultures were harvested after 17 hr of growth in either medium I or II. The pellet from 10 ml of culture was washed with 5 ml of transformation medium and resuspended in 3 ml of transformation medium; suspended cells were then diluted 20 times into transformation medium plus NaCl. A 20-ml amount of the diluted cells was shaken at 37 C in a 250-ml Erlenmeyer flask for 170 min. Samples of 0.4 ml were then pipetted into tubes and shaken for 10 min at 37 C. At 180 min, DNA was added, and 40 min later dilutions for plating were made into tubes (at 45 C) containing 3 ml of agar overlay. The first dilution tube for samples subjected to osmotic shock contained deionized water (at 45 C) instead of agar overlay.

<sup>b</sup> For culture I, guanosine replaced adenosine as growth supplement in both growth and transformation media. Culture II contained guanosine monophosphate instead of adenosine in both media. Transformation medium for both cultures contained one-tenth volume of CaCl<sub>2</sub>.

<sup>c</sup> The figures in parentheses indicate per cent loss due to the osmotic shock procedure.

ably decreased (48 and 32%) in both cultures which were subjected to the water dilution step.

A culture of *B. subtilis* 168 try<sup>-</sup> transformed by the method of Anagnostopoulos and Spizizen (1) and osmotically tested as outlined above gave results quantitatively similar to the more sensitive population (culture II) of *B. licheniformis*.

Transformation of *B. licheniformis* resembles that of *B. subtilis* in that transformed cells appear to be in a "dormant" state with respect to cell division. Figure 3 shows plate counts of Ad<sup>+</sup> transformants and total viable cells obtained from a competent culture which was exposed to transforming DNA at 360 min, and 15 min later was diluted 40 times into fresh transformation medium lacking adenosine and containing 100  $\mu$ g of deoxyribonuclease per ml. Although the bulk of the population was capable of continued cell division under these conditions, Ad<sup>+</sup> transfor-

ants did not begin to divide until at least 160 min after exposure to DNA was terminated.

Earlier reports on *B. licheniformis* transformation indicated that DNA concentrations in the range of 10 to 50  $\mu$ g/ml were required for saturation, and the majority of reported experiments were carried out at this relatively high range. However, the more highly purified DNA preparations used in this study routinely showed saturation concentrations of 0.1 to 0.2  $\mu$ g of DNA per ml, a finding similar to those in the *B. subtilis* and other bacterial transformation systems. Figure 4 represents a DNA-response curve of *B. licheniformis* transformation obtained with DNA purified as described in Materials and Methods.

To determine what molecular weight species, if any, present in DNA isolated as described in Materials and Methods was most effective in *B. licheniformis* transformation, a fractionation was carried out on a Bio-gel A-50m column. The product literature indicates that this type of column material fractionates in the molecular

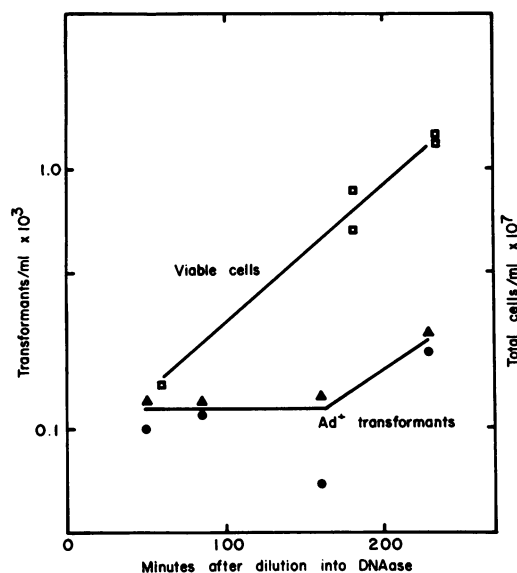


FIG. 3. Division of cells following transformation. A 20-hr growth culture of Ad<sup>-</sup> recipient was harvested by centrifugation. The pellet from 10 ml of culture was washed in 5 ml of transformation medium, resuspended in 3 ml of transformation medium, and diluted 50 times into transformation medium plus NaCl. Samples of 0.45 ml of diluted culture were pipetted into each of three tubes which were then shaken at 37 C to develop competence. At 360 min, DNA (two different preparations) was added to two of the tubes, and 15 min later samples from all three tubes were diluted 40 times into transformation medium plus NaCl (adenosine omitted) containing 100  $\mu$ g of deoxyribonuclease per ml. Shaking at 37 C was continued, and Ad<sup>+</sup> transformants were determined at intervals in the two tubes exposed to DNA. Viable-cell counts were made on the control tube without added DNA.

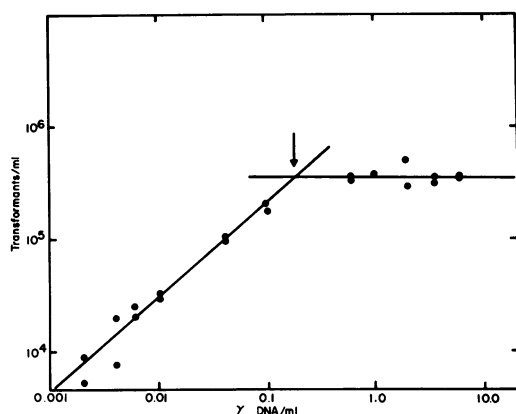


FIG. 4. Transformation as a function of DNA concentration. A culture was harvested after 17 hr of growth. (Growth medium and transformation medium contained 20  $\mu$ g of guanosine per ml in addition to 20  $\mu$ g of adenosine per ml. CaCl<sub>2</sub> concentration of transformation medium was decreased to one-tenth of that given in Materials and Methods.) The pellet from 10 ml was washed with 5 ml of transformation medium and resuspended in 3 ml of transformation medium; the resuspended cells were diluted 20 times into transformation medium plus NaCl. A 50-ml amount of diluted culture was shaken at 37 C in a 500-ml Erlenmeyer flask. At 200 min, 0.4-ml portions of culture were pipetted into tubes on a shaker at 37 C, containing various amounts of DNA in a total volume of 0.1 ml. Dilutions for plating were carried out 35 min after addition of cells to DNA. Transformants per milliliter are plotted against micrograms of DNA per milliliter present in tube during transformation. (Transformation obtained at saturation concentrations of DNA was 0.87%).

weight range of  $10^6$  to  $5 \times 10^7$ . Since almost all of the material absorbing at 260 nm in the DNA preparation applied to the column eluted within the first half of the bed volume, the DNA was roughly estimated to contain molecules with a molecular weight between  $8 \times 10^6$  and  $5 \times 10^7$ . Column tubes were combined as shown in Fig. 5 to give four fractions (I through IV), each containing DNA of a different average molecular weight. Tests of the biological activity of these four fractions at two concentrations below saturation (0.03 and 0.05  $\mu\text{g}$  of DNA per ml) indicated that size of the DNA (within the  $8 \times 10^6$  to  $5 \times 10^7$  molecular weight range present in this preparation) had little effect on its ability to transform. As is shown in the upper section of Fig. 5, the numbers of  $\text{Ad}^+$  transformants per  $\mu\text{g}$  of DNA were very similar in the four fractions tested.

Melting curves were carried out on DNA present in fractions I through IV of the experiment described above. The  $T_m$  and per cent hyperchromicity of each of the fractions were virtually identical with those of unfractionated DNA. Unfractionated DNA from this  $\text{Ad}^+$   $\text{Cap}^-$  auxotroph of *B. licheniformis* 9945A had a  $T_m$  of 87.8 C (in standard saline-citrate), in very close agreement with the  $T_m$  of 87.5 C found for *B. subtilis* DNA (10).

The lower saturation concentration of DNA required in transformation of *B. licheniformis* with the methodology of the present report suggested the possibility that some inhibitory contaminant might have been present in less highly purified DNA used in earlier investigations. Because methods of DNA preparation as described by Gwinn and Thorne (5) involved procedures in which protein was removed but no digestion with ribonuclease was carried out, it was considered that contaminating ribonucleic acid (RNA) might be implicated. However, when DNA free from RNA (prepared as described in Materials and Methods) was compared in transforming activity with DNA containing up to 50% of its material absorbing at 260 nm as RNA (prepared as described by the authors cited above), the two preparations proved to have identical DNA saturation curves. RNA, therefore, was not an inhibitory contaminant of major significance.

To test the possibility that the methodology presented here for an  $\text{Ad}^-$  auxotroph of *B. licheniformis* might specifically favor transfer of that particular genetic locus, transformation of another marker was tested. Preparation of multiply marked auxotrophs from the  $\text{Ad}^-$  recipient strain might introduce unknown alterations in loci affecting development of competence, such as those governing capsule formation. It was therefore decided to study transfer of a strepto-

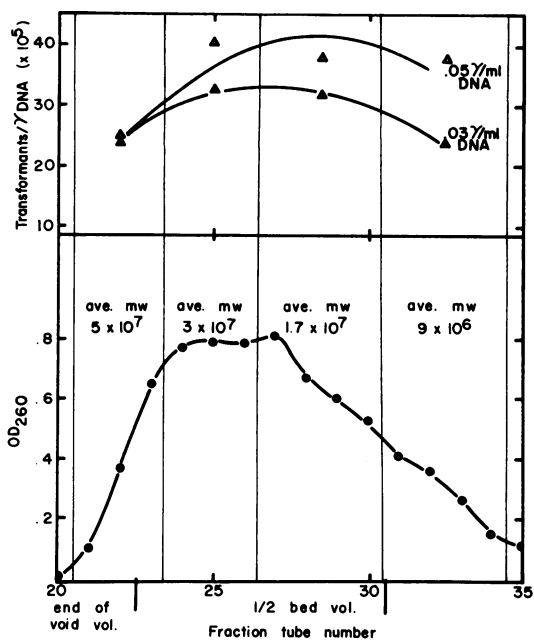


FIG. 5. Fractionation of transforming DNA on Bio-gel A-50m and biological activity of DNA fractions. DNA was prepared from the  $\text{Ad}^+$  transformant as indicated in Materials and Methods, and was stored in 2.0 M NaCl. Approximately 3 ml of DNA (estimated to contain approximately 750  $\mu\text{g}$  of DNA) was applied to a Bio-gel A-50m column (2.0 cm diameter  $\times$  31 cm height) previously equilibrated with 0.5 M NaCl. Elution was carried out with 0.5 M NaCl, and 1.6-ml fractions were collected. Optical density readings at 260 nm of the fractions are plotted against tube number. To determine the biological activity of DNA fractions, pooled fractions I to IV from column fractionation A were appropriately diluted (assuming that an optical density at 260 nm of 1.0 = 45  $\mu\text{g}$  of DNA per ml) to give final concentrations in transformation tubes of either 0.03 or 0.05  $\mu\text{g}$  of DNA/ml. The culture of recipient cells was harvested after 18 hr of growth, washed with 5 ml of transformation medium, resuspended in 3 ml of transformation medium, and diluted 50 times into transformation medium plus NaCl. Tubes containing 0.45 ml of suspended cells were shaken for 250 min at 37 C, and DNA from fractions I to IV was added. Dilution and plating for viable and  $\text{Ad}^+$  transformants was carried out 60 min later. (Per cent transformation obtained varied between 0.12 and 0.26%). Transformants per microgram of DNA are plotted over the appropriate fraction for the two DNA concentrations used.

mycin-resistance trait ( $\text{Sm}^r$ ). DNA for these  $\text{Sm}^r$  studies was isolated from an  $\text{Sm}^r$  mutant obtained by ultraviolet radiation of the same  $\text{Ad}^-$  auxotroph used as recipient in these experiments. In controls where  $\text{Ad}^+$  transformation was determined, DNA was obtained from the  $\text{Ad}^+$   $\text{Cap}^-$  transformant as previously described. When

a competent culture of the  $\text{Ad}^-$  recipient was exposed for 35 min to 1 to 2  $\mu\text{g}$  of DNA per ml from donor cells containing either the  $\text{Ad}^+$  or  $\text{Sm}^r$  marker as outlined above, 1.75% transformation for  $\text{Ad}^+$  and 1.35% transformation for  $\text{Sm}^r$  were obtained. The same  $\text{Ad}^-$  recipient strain was used in studies of  $\text{Sm}^r$  reported by Goldberg, Gwinn, and Thorne (4). Their data (obtained with the methodology of Leonard et al. which involves 3 to 4 hr of exposure to DNA) indicated that exposure to 10 to 20 times this concentration of DNA (22  $\mu\text{g}/\text{ml}$ ) produced 1.23% transformation for  $\text{Ad}^+$  and 0.25% transformation for  $\text{Sm}^r$ . The system reported here thus appears to be a general one, applicable to transfer of various genetic markers and with increased efficiency relative to methods previously used.

### DISCUSSION

Transformation of an  $\text{Ad}^-$  auxotroph of *B. licheniformis* by the modified techniques described in this report is similar to that observed in the closely related organism, *B. subtilis* 168, in the following respects. (i) A "step-down" during transfer of cultures from growth to transformation medium appeared to be effective in producing more competent cultures. (ii) A period of several hours after transfer to the step-down medium was required before maximal competence was reached. (In the case of *B. licheniformis*, this period appeared to be related to initial cell density.) (iii) Newly transformed cells were in a "dormant" state as measured by cell division. (iv) Newly transformed cells were less able to recover from the damage caused by osmotic shock than were noncompetent cells. (v) Approximately 0.1  $\mu\text{g}$  of DNA/ml was the saturating concentration for transformation. (vi) No difference in biological activity was found in molecular weight species of DNA within the range  $8 \times 10^6$  to  $5 \times 10^7$ . (vii) The modified transformation system presented here appears to be applicable to the transfer of at least two genetic markers. Equally efficient transformation of two different markers,  $\text{Sm}^r$  and  $\text{Ad}^+$ , was obtained.

Data presented in this report show transformation frequencies in the range of 0.1 to 1.0%. Some of the experiments illustrated were carried out under less than optimal conditions in order to study variables of the system, and it should be emphasized that a range of 1.0 to 3.0% transformation can be routinely obtained, even with relatively short times of exposure to DNA (15 to 30 min), provided the variables studied here are rigorously controlled. No attempt was made to determine the maximal transformation obtainable

with the extended times of exposure to high concentrations of DNA used by other investigators, since such conditions were not considered useful in a system for metabolic or genetic studies.

Transforming DNA for these studies was prepared from an  $\text{Ad}^+$   $\text{Cap}^-$  transformant of the recipient culture. This procedure was carried out in order to avoid capsule formation on plates by  $\text{Ad}^+$   $\text{Cap}^+$  transformants obtained when DNA from the wild-type parent strain was used. If traces of capsular material present in such wild-type DNA are inhibitory to transformation, then the use of  $\text{Cap}^-$  DNA in these experiments may provide an explanation for the lower DNA saturation curves obtained.

It has been shown (9) that capsule formation in *B. licheniformis* is inversely related to ability to be transformed. Some extremely interesting papers by Leonard et al. (6, 7) have also indicated that there are several loci governing the relative production of D- or L-glutamic acid-containing peptides of capsular material, and the expression of these loci is differentially affected by salt composition in the growth medium. These facts taken together suggest that the varied salt requirements for optimal transformation of different auxotrophs from the same parent strain may reflect the presence of different capsule loci. Transformable auxotrophs of *B. licheniformis* isolated by Leonard are all classified  $\text{Cap}^-$  relative to visible capsule production by colonies of the wild type (plated under conditions optimal for wild-type capsule formation). Leonard has, however, demonstrated (8) that these various auxotrophs all classified as "nonencapsulated" in fact contain a variety of capsule loci. Under other growth conditions, some of these loci may be capable of expression, producing either minute amounts of normal capsule or an altered material. The M 28 glycine $^-$  auxotroph of *B. licheniformis* 9945A used by Gwinn and Thorne is a case in point. This auxotroph is  $\text{Cap}^-$  on plates of minimal medium when compared with wild type but under other nutritional conditions is  $\text{Cap}^+$ , merely producing less capsular polypeptide than does the wild type (5). It is assumed that the media and techniques used here in transformation of an  $\text{Ad}^-$  auxotroph are those affecting capsule loci in such a way as to produce wall or capsule formation most favorable to competence. If this assumption is correct, then the system is not likely to prove directly applicable to other auxotrophs where different capsule loci are present. It would, however, be predicted that this methodology should be optimal for any multiply marked auxotrophs prepared from this  $\text{Ad}^-$  auxotroph by techniques in which capsule loci are not affected.

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